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Patent- und Rechtsanwälte

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(54) **PROCESS FOR PRODUCING COENZYME Q10**

(57) A gene of decaprenyl diphosphate synthase, which is the key gene participating in the biosynthesis of coenzyme Q₁₀ was isolated from a bacterium belonging to the family *Rhizobiaceae*. By transferring this gene into a microorganism such as *Escherichia coli* and expressing therein, coenzyme Q₁₀ can be effectively produced.

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Description

Technical Field

[0001] The present invention relates to a process for producing coenzyme Q₁₀, which can be used as a pharmaceutical agent and the like. In more detail, the present invention relates to a process for producing coenzyme Q₁₀ by isolating a gene coding for an enzyme responsible for biosynthesizing coenzyme Q₁₀ side chain, the key enzyme in the coenzyme Q₁₀ biosynthetic pathway, i.e. decaprenyl diphosphate synthase, from a bacterium belonging to the family *Rhizobiaceae*, and transferring said gene into a microorganism to produce coenzyme Q₁₀.

Background Art

[0002] Industrially, coenzyme Q₁₀ has been produced by isolating a coenzyme from plants such as tobacco and synthetically altering the side chain.

[0003] In addition, it has been known that coenzyme Q₁₀ is produced by wide variety of organisms including from microorganisms such as bacterium and yeast, to higher plants and animals. One of the most effective processes for producing coenzyme Q₁₀ is believed to be a process which comprises culturing the microorganism and extracting the compound from the culture. Said process has also been used in industrial production of coenzyme Q₁₀. However, the above known processes do not provide enough productivity because of their low yield or complicated operation.

[0004] Though there are some differences between the prokaryotic and eucaryotic pathways for biosynthesis of coenzyme Q₁₀, both pathways are consisted of complicated multi-step reactions in which many enzymes are involved. They are basically consisting of the following three steps; synthesizing decaprenyl diphosphate, which is used for the prenyl side chain of coenzyme Q₁₀; synthesizing parahydroxy benzoic acid, which is used for the quinone ring; and binding those two components and converting the substituents sequentially to give coenzyme Q₁₀. The reaction, which determines the length of the side chain and is believed to be the rate-limiting step in the biosynthetic pathway, that is, the reaction in which decaprenyl diphosphate synthase is involved, is believed to be the most important reaction. Therefore, in order to produce coenzyme Q₁₀ effectively, it might be a good idea to isolate decaprenyl diphosphate synthase gene, the key gene in the biosynthetic pathway, and to use said gene for improving the productivity. One of the potential candidates for the gene sources is a bacterium belonging to the family *Rhizobiaceae*, which produces relatively large amount of coenzyme Q₁₀.

[0005] Until now, decaprenyl phosphoric acid synthase genes had been isolated from several microorganisms including *Schizosaccharomyces pombe*

(Japanese Patent Application Laid Open No. H09-173076) and *Gluconobacter suboxydans* (Japanese Patent Application Laid Open No. H10-57072.) However, they do not show enough productivity for coenzyme Q₁₀ and therefore, the art had not yet achieved effective culture, isolation or purification with those microorganisms. Accordingly, it has been desired to isolate a gene of said enzyme from a microorganism having high coenzyme Q₁₀ producing ability.

Problems to be solved by the Invention

[0006] The present invention has been made to solve the above-mentioned problem of less productivity. The object of the present invention is to provide a process for producing coenzyme Q₁₀ effectively by means of a microorganism, by isolating a gene coding for coenzyme Q₁₀-side chain synthetic enzyme from a bacterium belonging to the family *Rhizobiaceae* and using the gene.

[0007] According to the present invention, decaprenyl diphosphate synthase gene, the key gene in the biosynthetic pathway for coenzyme Q₁₀, was isolated from a bacterium belonging to the family *Rhizobiaceae*. An effective coenzyme Q₁₀ production has been achieved by transferring the gene into a microorganism such as *Escherichia coli* and expressing therein.

Summary of the Invention

[0008] The inventors have tried to isolate the gene of decaprenyl diphosphate synthase from a bacterium belonging to the family *Rhizobiaceae* which produces relatively large amount of coenzyme Q₁₀ and succeeded to isolate said gene.

[0009] Accordingly, the present invention provide a DNA comprising a DNA sequence of the Seq. ID No. 1, or a sequence having deletion, addition or insertion of one or more base in the sequence and coding for decaprenyl diphosphate synthase. The present invention also provide a protein having an amino acid sequence of the Seq. ID No. 2, or an amino acid sequence having deletion, addition or insertion of one or more amino acid in said sequence and having decaprenyl diphosphate synthase activity; and a DNA encoding said amino acid sequence.

[0010] The present invention also provide a process for producing coenzyme Q₁₀ comprising the steps of transferring the above described DNA sequence into a host microorganism and culturing the microorganism. The host microorganism used in the present invention is not limited but preferably is *Escherichia coli*. Although a normal *Escherichia coli* produces coenzyme Q₈, according to the present invention, *Escherichia coli* can be modified to produce coenzyme Q₁₀.

[0011] In addition, the present invention provides an expression vector comprising the above-described DNA sequence. The expression vector of the present inven-

tion may be constructed by using any of known vector systems. For example, pQAD-1, which is constructed by transferring the gene of the Seq. ID No. 1 into the known expression vector system pUCNT, is provided.

[0012] According to the invention, a host microorganism transformed with the above-described DNA sequence is also provided. For the host microorganism in the present invention, *Escherichia coli* is preferably used.

Brief description of the Drawings

[0013]

Fig. 1 shows a restriction map of plasmid pQAD1 which contains decaprenyl diphosphate synthase gene.

Fig. 2 shows a chart of high-speed liquid chromatography detecting coenzyme Q₁₀ produced by recombinant *E.-coli* comprising decaprenyl diphosphate synthase gene.

Best Mode for Carrying out the Invention

[0014] The inventors studied intensively to isolate the desired gene from the bacterium belonging to the family *Rhizobiaceae*, which produces relatively high amount of coenzyme Q₁₀, and succeeded to obtain a fragment of said gene by means of PCR technique.

[0015] The inventors compared known decaprenyl diphosphate synthase genes and polyprenyl diphosphate synthase genes, analogous enzymes of the former that participate biosynthesis of longer prenyl chains to provide coenzyme Qs having different length side chains; and based on the homologous regions between those sequences, several PCR primers were designed. Various combinations of the obtained primers were tested to determine PCR condition. It was found that a 400 bp gene fragment of the desired enzyme was amplified from chromosomal gene of *Agrobacterium sp.* KNK712 (FERM BP-1900) by using DPS-1 (5'-AAG-GATCCTNYTNCAYGAYGAYGT-3') and DPS-2 (5'-AAGGATCCTCRTCACNARYTGRAA-3') (wherein R represents A or G, Y represents C or T, and N represents G, A, T or C) as PCR primers, carrying out the PCR process at 94 °C for 1 minute then 26 cycles of thermal cycling at 94°C 1min. → 50°C 1 min. → 75°C 1 min. It was confirmed by sequencing the obtained gene.

[0016] In order to obtain full length gene of said enzyme, in the next step, *Agrobacterium sp.* KNK 712 (FERM BP-1900) chromosomal gene was digested with EcoRI restriction enzyme and the obtained fragments were transferred into λ phage vector to provide a recombinant phage library. The plaques were blotted on a nylon filter and the filter was subjected to plaque hybridization with the labeled PCR fragment and then, a clone comprising full length of decaprenyl diphosphate synthase gene could be obtained.

[0017] Base sequence of the decaprenyl diphosphate synthase gene contained in the obtained clone was determined to give the sequence of the Seq. ID No. 1. The amino acid sequence deduced from the base sequence contained regions having sequences characteristic to decaprenyl diphosphate synthase.

[0018] In order to express the decaprenyl diphosphate synthase gene, it is required to ligate said gene into a vector at a region downstream to an appropriate promoter. For example, an expression vector may be constructed by excising a DNA fragment containing desired gene by means of restriction enzyme or by amplifying the fragment coding for the enzyme by means of PCR, then transferring the fragment into a vector having a promoter. For example, an expression vector system pUCNT (described in WO94/03613) may be transfected with said gene to provide an expression vector pQAD1 for decaprenyl diphosphate synthase gene.

[0019] Then, an appropriate microorganism may be used for producing coenzyme Q₁₀ by transforming the same with said expression vector for the enzyme. For example, a *Escherichia coli*, which produces originally coenzyme Q₈, can be transformed by pQAD1, the expression vector for decaprenyl diphosphate synthase gene, to produce significantly higher amount coenzyme Q₁₀, which is not originally produced, than the amount of coenzyme Q₈ production.

[0020] The transformed *Escherichia coli*, *Escherichia coli* HB101 pQAD1 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry with the accession number of FERM BP-6538.

[0021] The gene provided by the present invention may be utilized alone or may also be co-transferred into a microorganism with another biosynthetic gene and expressed therein to give a better effect.

[0022] According to the process of the present invention for producing coenzyme Q₁₀, the host microorganism transformed with the gene may be cultured to produce coenzyme Q₁₀. The condition for the culture is not limited and may be determined depending the selected host microorganism. Conditions for culturing various host microorganisms are well known to the art. After the culture is completed, the host microorganisms may be harvested and coenzyme Q₁₀ may be isolated and purified by means of an appropriate procedure. The method for isolating coenzyme Qs from the host microorganism is well known to the art.

Examples

[0023] The present invention will be described in more detail by means of the following examples. The examples are for the purpose of explanation only and do not limit the scope of the invention in any means.

(Example 1)

[0024] A chromosomal DNA of *Agrobacterium sp.* KNK712 was prepared by the method of Marmur, J. Mol. Biol. Vol. 3, pp 208-218 (1961). PCR primers were designed based on base sequence homology between the DNA and the known long chain prenyl phosphoric acid synthase gene, to give two primers DPS-1 (5'-AAGGATCCTNYTNCAYGAYGT-3') and DPS-2 (5'-AAGGATCCTCRTCACNARYTGRAA-3'). Wherein, R represents A or G, Y represents C or T, and N represents G, A, T, or C. They were subjected to the PCR thermal cycle (94°C 1min → (25 cycles at 94°C 1min. → 50°C 1 min. → 70°C 1min) → 4°C 1 min. The amplified mixture was analyzed by 0.8% agarose gel electrophoresis. A 400 bp fragment was excised from the gel and purified by means of DNA extracting kit (Takara Co.), and then DNA base sequence of the fragment was determined by means of DNA sequencer (373 A type, Appliedbiosystems Co.) with DNA sequence kit (ABI PRISMTM Dye Terminator Cycle Sequence Ready Reaction Kit with Amplitaq^R DNA polymerase, FS) according to the supplier's instruction. As a result, a base sequence corresponding to nucleotides 514 to 905 of the Seq. ID No. 1 was obtained. The amino acid sequence translated from the base sequence had regions "VGDFLLG" and "EGEVLLQ" which were characteristic to the synthetic enzyme of prenyl diphosphate having long prenyl chain. Accordingly, we confirmed the obtained gene was a part of decaprenyl diphosphate synthase gene.

(Example 2)

[0025] *Agrobacterium sp.* KNK712 chromosomal DNA (0.25 µg) was subjected to PCR amplification with primers NQE-11 (having the sequence of 5'-AAGTC-CACCGCCCGCAGATCT-3') and NQE-12 (having the sequence of 5'-CCGAGGTTTCATGCCGTAGGATTTT). The PCR was carried out at 94°C 1min → (25 cycles at 94°C 1min. → 50°C 1min. → 70°C 1min) → 4°C 1min. The amplified mixture was separated by 0.8% agarose gel electrophoresis, an about 320 bp fragment was excised from the gel and then the fragment was purified by means of DNA extracting kit (Takara Co.). 25ng of the obtained DNA fragment was labeled with [α -32P]dCTP by means of MegaprimeTM DNA labeling system (Amersham Co.),

(Example 3)

[0026] *Agrobacterium sp.* KNK712 chromosomal DNA was digested with restriction enzymes EcoRI, Sac I, Not I and Xho I, and separated by 0.8% agarose gel electrophoresis. Then, the gel was denatured by means of alkaline solution (0.5M NaOH, 1.5M NaCl) and neutralized (0.5M Tris · HCl(pH 7.5)). Highbond N+ filter (Amersham Co.) was placed on the gel and the DNA

bands on the gel were Southern transferred to the filter with 10 × SSC. The obtained filter was dried and fixed at 80 °C for 2 hours, and then, the filter was prehybridized at 60°C, for 4 hours in the prehybridizing solution (15ml of 20 × SSC(3M NaCl, 0.3M trisodium citrate dihydrate, pH 7.0), 5ml of 10% SDS(sodium dodecyl sulfate), 5ml of 50 × Denhardt's solution(10g/l Ficoll Type 400, (Pharmacia), 10g/l of polyvinylpyrrolidone and 10g/l bovine serum albumin (Fraction V, Sigma)) 0.5ml of 10mg/ml salmon sperm DNA (denatured by heating at 95 °C for 5minutes and quenched on ice) and 24.5ml of water).

[0027] The labeled probes were heated at 95 °C for 5 mm and quenched on ice, and were added into the prehybridizing solution containing the prehybridized filter. Hybridization was carried out at 60°C for 22 hours. The hybridized filter was washed two times at room temperature with 5 × SSC supplemented with 0.5% SDS, and then with 1 × SSC supplemented with 0.1% SDS while the temperature was gradually rose from 60°C to 75°C. The filter was dried and exposed by adhering to the X-ray film, and then black bands were developed.

[0028] As a result, the probes were strongly hybridized with EcoRI fragment of about 2.7kb, Sac I fragment of about 4.7 kb, Not I fragment of about 8.3 kb, and Xho I fragment of about 4.7kb.

(Example 4)

[0029] A chromosomal DNA of *Agrobacterium sp.* KNK 712 was cut with EcoRI restriction enzyme, separated by 0.8% agarose gel electrophoresis, then a DNA fragment of approx. 7kb was excised and purified to give the DNA fragment for cloning. The obtained DNA fragment was inserted into the λ -ZAP RII phage kit (Stratagene Co.) at EcoRI site and packaged using in vitro packaging kit (Amersham Co.). Then, E-coli XL 1-Blue MRF⁺ was infected with the phage. The infected cells were plated onto NZY plate culture medium (5g/l NaCl, 2g/l MgSO₄·7H₂O, 5g/l yeast extract, 10g/l NZ amine, 18g/l agar (pH 7.5) together with NZY soft agar medium (same as NZY plate culture medium except for the amount of agar is 8g/l) and cultured to develop plaques. The plaques were transferred on to the Highbond N+ filter (Amersham Co.). The filter was denatured with an alkaline solution (0.5M NaOH, 1.5M NaCl), neutralized (0.5M Tris · HCl(pH 7.5), 1.5M NaCl), dried and fixed at 80°C for 2 hours.

[0030] Twenty-four filters obtained as above were prehybridized and then hybridized with labeled probes according to the similar procedure of Example 3, and the obtained filters were washed. After dried, the filters were exposed to X-ray by adhering to the X-ray films. Plaques of the phages corresponding to the black spots developed were isolated. The isolated phages were infected into E-coli according to the above-described method. The obtained plaques were blotted onto the filter and again hybridized to confirm. Twelve strains of the

phage were selected.

[0031] PCR was carried out using suspension of the respective phages and the aforementioned NQE-11 and NQE-12. 320bp DNA fragments were found in eight strains of the 8 using λ -ZAP RII Phage Kit according to the supplier's instruction.

(Example 5)

[0032] Using thus obtained two phagemid DNAs, DNA base sequence of decaprenyl diphosphate synthase gene was determined according to the process similar to example 1. Among the inserted DNAs, a DNA base sequence fragment of about 1.6 kb were determined. The obtained sequence is shown in the sequence listings as the Seq. ID No. 1. An amino acid sequence deduced from the base sequence is shown as the Seq. ID No. 2.

[0033] The obtained sequences were compared with those of decaprenyl diphosphate synthase derived from *Gluconobacter suboxydans* (Japanese Patent Application Laid Open H10-51072) and found that they showed about 47% of amino acid sequence homology and about 60% of DNA sequence homology. The results are shown in Figs 3, 4 and 5. The sequences were also compared with decaprenyl diphosphate synthase derived from *Schizosaccharomyces pombe* and found that they showed about 30 % amino acid and 46% DNA homologies.

(Example 6)

[0034] In order to excise the coding region for decaprenyl diphosphate synthase from the above prepared phagemid, PCR was carried out using synthetic DNA primers NQE-22 (having the sequence of 5'-AGT-CAAGCTTCAGCTCACCCGGTCGATC-3') and NQE-23 (having the sequence of 5'-AGCTCATATGATAC-CGCTGGAA GACAGC-3') according to the same procedure as example 3. The obtained fragment was cut with NdeI and Hind III restriction enzymes and the obtained fragment was transferred into the expression vector pUCNT(WO94/03613) to give expression vector pQAD1 for the decaprenyl diphosphate. The restriction map of pQAD1 is shown in Fig. 1. In this figure, DPS represents the coding region of the decaprenyl diphosphate synthase.

(Example 7)

[0035] Thus prepared expression vector for decaprenyl diphosphate synthase was added to E-coli HB101, the cells were shaken over night in 10 ml LB medium at 37°C and then, collected (3000 rpm, 20 min.).

[0036] The collected cells were suspended into 1ml of 3% aqueous sulfuric acid, heated to 120°C for

30minutes, added with 2ml of 14% aqueous sodium hydrate and heated to 120°C for additional 15 minutes. Thus treated mixture was extracted by adding 3ml of hexane/isopropanol (10:2) and centrifuged. 1.5ml of the organic solvent phase was separated and evaporated to dryness under reduced pressure. The residue was dissolved in 0.5ml ethanol and 20 μ l of the solution was analyzed with high speed liquid chromatography system (LC-10A; Shimadzu Co.). For elution, reverse phased column (YMC-pack ODS-A, 250 \times 4.6 mm, S-5 μ m, 120A) was used and ethanol/methanol (2:1) was used as a mobile phase solvent. The produced coenzyme Q₁₀ was monitored by absorbance at 275 nm wavelength. Results are shown in Fig. 2. As is apparent from Fig. 2, the recombinant *Escherichia coli* obtained by introducing and expressing decaprenyl diphosphate synthase gene produce coenzyme Q₁₀, which is not produced by wild type strain.

[0037] The obtained recombinant E-coli strain, *Escherichia coli* HB101 pQAD1 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on October 1, 1996, (Accession No. FERM BP-6538.)

Industrial Applicability

[0038] In the present invention, a gene coding for decaprenyl diphosphate synthase, the key enzyme in the coenzyme Q₁₀ biosynthetic system, was isolated from the bacterium belonging to the family *Rhizobiaceae* and the nucleotide sequence was determined. In addition, the inventors succeeded to introduce the obtained gene into *Escherichia coli* and to express the same. By using the gene and the process of the present invention, coenzyme Q₁₀, which can be used for manufacturing pharmaceutical compositions and the like, can be prepared effectively.

Reference to the deposited microorganisms

[0039] *Escherichia coli* HB101 pQAD1 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on October 1, 1996. The accession No. FERM BP-6538 was assigned.

Claims

1. A DNA which comprises, a base sequence of the Seq. ID No. 1, or a base sequence having one or more deletion, addition or insertion in the sequence of the Seq. ID No. 1 and coding for decaprenyl diphosphate synthase.
2. A DNA encoding an amino acid sequence which comprises, an amino acid sequence of the Seq. ID

- No. 2, or an amino acid sequence having one or more amino acid deletion, addition or insertion in the Seq. ID No. 2 and having a decaprenyl diphosphate synthase activity.
3. A protein which comprises, an amino acid sequence of the Seq. ID No. 2, or an amino acid sequence having one or more amino acid deletion, addition or insertion in the Seq. ID No. 2 and having a decaprenyl diphosphate synthase activity.
4. A process for producing coenzyme Q₁₀ which comprises the steps of, transferring a DNA of the Seq. ID No. 1, or a DNA having one or more base deletion, addition or insertion in the Seq. ID No. 1 and coding for decaprenyl diphosphate synthase into a host microorganism and culturing the host microorganism.
5. A process for producing coenzyme Q₁₀ which comprises the steps of, transferring a DNA encoding an amino acid sequence of the Seq. ID No. 2, or an amino acid sequence having one or more amino acid deletion, addition or insertion in the Seq. ID No. 2 and having a decaprenyl diphosphate synthase activity, into a host microorganism and culturing the host microorganism.
6. The process according to claim 4 or 5, wherein the host microorganism is *Escherichia coli*.
7. The process according to claim 6, which comprises the step of culturing *Escherichia coli* HB101 pQAD1 (FRPM BP-6538).
8. An expression vector which comprises, a DNA comprising a base sequence of the Seq. ID No. 1, or a base sequence having one or more base deletion, addition or insertion in the Seq. ID No. 1 and coding for decaprenyl diphosphate synthase.
9. An expression vector which comprises, a DNA encoding an amino acid sequence of the Seq. ID No. 2 or an amino acid sequence having one or more amino acid deletion, addition or insertion in the Seq. ID No. 2 and having a decaprenyl diphosphate synthase activity.
10. Expression vector pQAD1, which is obtainable by transferring a DNA sequence into an expression vector pUCNT.
11. A host microorganism which is transformed with a DNA comprising a base sequence of the Seq. ID No. 1, or a base sequence having one or more base deletion, addition or insertion in the Seq. ID No. 1 and coding for decaprenyl diphosphate synthase.
12. A host microorganism transformed with a DNA encoding an amino acid sequence of the Seq. ID No. 2, or an amino acid sequence having one or more amino acid deletion, addition or insertion in the Seq. ID No. 2 and having a decaprenyl diphosphate synthase activity.
13. The microorganism according to claims 11 or 12, which is obtainable by transforming *Escherichia coli*.
14. The microorganism according to claim 13, which is *Escherichia coli* HB 101 pQAD1 (FERM BP-6538.)

Fig. 1

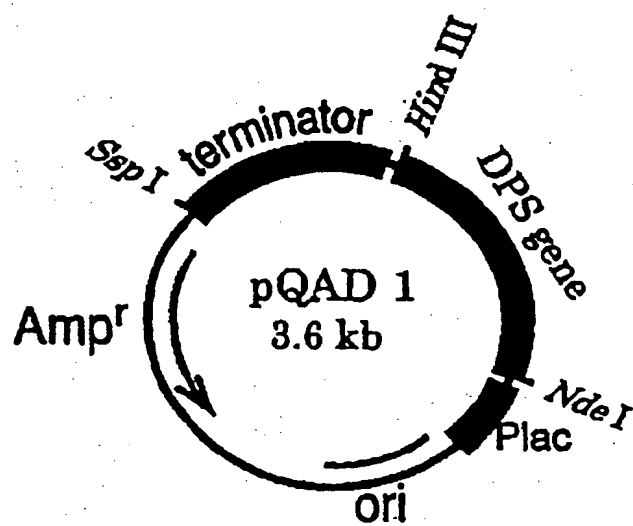
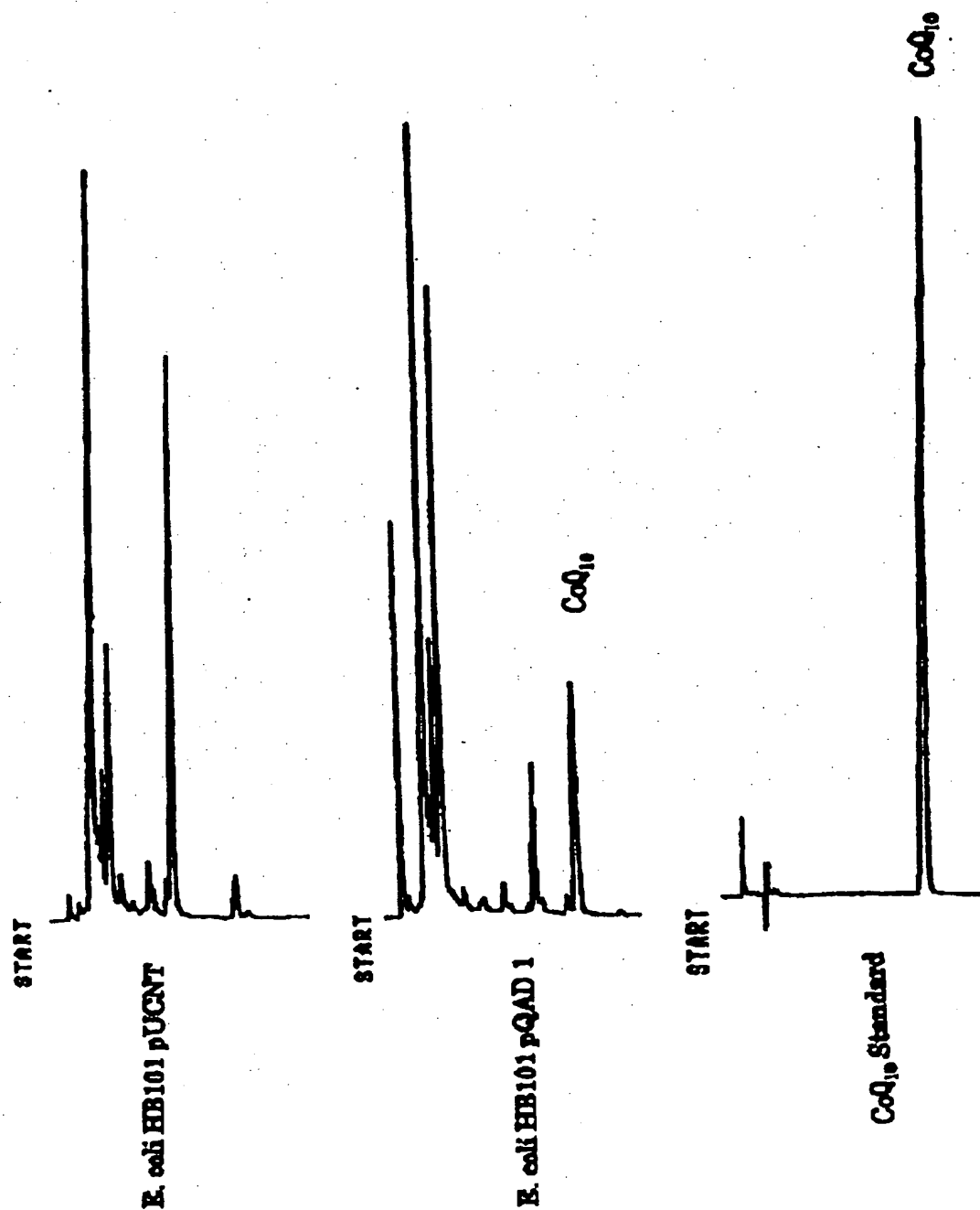


Fig. 2



INTERNATIONAL SEARCH REPORT

International application No.

PCT/JP00/00588

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl⁷ C12N 15/52, C12N 9/52, C12N 1/21// (C12N 15/52, C12R 1:19), (C12N 9/52, C12R 1:19), (C12N 1/21, C12R 1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl⁷ C12N 15/00-90, C12N 9/00-99, C12N 1/00-38

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE (STN), Genbank/EMBL/DDBJ/GeneSeq,
WPI (DIALOG), BIOSIS (DIALOG)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Eur. J. Biochem., (1998), Vol.225, No.1, p.52-59, Matsuda H. et al., "Molecular cloning and mutational analysis of the ddaA gene encoding decaprenyl diphosphate synthase from Gluconobacter suboxydans"	1-14
A	J. Biochem., (1997), Vol.121, No.3, p.496-505, Kawamukai M. et al., "Analysis of the decaprenyl diphosphate synthase (dps) gene in fission yeast suggests a role of ubiquinone as an antioxidant",	1-14
PA	JP, 11-178590, A (TOYOTA JIDOSHA KK), 06 July, 1999 (06.07.99) (Family: none)	1-14
PA	JP, 11-056372, A (ALPHA SHOKUHIN KK), 02 March, 1999 (02.03.99) (Family: none)	1-14
A	JP, 10-057072, A (ALPHA SHOKUHIN KK), 03 March, 1998 (03.03.98) (Family: none)	1-14

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:
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